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TITLE

DETECTION OF CFTR PROTEIN IN HUMAN LEUKOCYTES BY FLOW CYTOMETRY

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ABSTRACT

Background: Leukocytes have previously been shown to express detectable levels of the protein Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). This study aims to evaluate the application of flow cytometry analysis to detect CFTR expression, and changes thereof, in these cells.

Methods: Aliquots (200 µL) of peripheral whole blood from; 12 healthy control volunteers (CTRLs), 12 carriers of a *CFTR* mutation (CFC) and 40 cystic fibrosis (CF) patients carrying various combinations of CFTR mutations, were incubated with specific fluorescent probes recognizing CFTR protein expressed on the plasma membrane of leukocytes. Flow cytometry was applied to analyze CFTR expression in monocytes, lymphocytes and in polymorphonuclear (PMN) cells.

Results: CFTR protein was detected in monocytes and lymphocytes whereas inconclusive results were obtained from analysis of PMN cells. MFI ratio value and %CFTR positive cells above a selected threshold were the two parameters selected to quantify CFTR expression in cells. Lowest variability and the highest reproducibility were obtained when analysing monocytes. ANOVA results indicated that both parameters were able to discriminate monocytes of healthy controls and CF individuals according to *CFTR* mutation classes with high accuracy. Significantly increased MFI ratio values were recorded in CFTR-defective cells that were able to improve also CFTR function after *ex vivo* treatment with PTC124 (Ataluren), an investigative drug designed to permit the ribosome to read-trough nonsense *CFTR* mutations.

Conclusions: The method described is minimally invasive and may be used in the monitoring of responses to drugs whose efficacy can depend on increased CFTR protein expression levels.

Key words

Cystic Fibrosis

Leukocytes

cystic fibrosis transmembrane conductance regulator (CFTR)

Genetic disease

INTRODUCTION

Cystic fibrosis (CF) is the most common recessively inherited disease among caucasians [1]. The affected gene encodes the protein Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), which functions as an anion channel in epithelial cells. Since its discovery in 1989, biomedical research has focused on targeting the underlying genetic defect to identify a disease-modifying therapy for CF patients. Over 1,900 mutations identified in the *CFTR* gene have been clustered in 5 major classes (available at http://www.genet.sickkids.on.ca/cftr/). Class I mutations impair CFTR protein synthesis, no CFTR protein reaches the plasma membrane and chloride transport through CFTR does not occur. Class II mutations cause subcellular mislocalization and rapid degradation of CFTR. The little CFTR protein that reaches plasma membrane often does not function properly. In class III and IV mutations CFTR is expressed on the plasma membrane but the chloride transport is impaired. Class V mutations have both reduced CFTR synthesis and a reduced number of CFTR channels that transport chloride through the plasma membrane appropriately [2]. A dysfunction of CFTR anion channel in epithelial cells is thought to be the main cause for hyperviscous mucus production, infections and inflammation in CF patients' lungs. However, excessive neutrophil accumulation in the lung is also a hallmark of CF disease and altered leukocyte functions have also been hypothesized to significantly contribute to the pathogenesis of the disease. Several data support this vision. Already back in 1991 Yoshimura et al. [3] demonstrated mRNA encoding for CFTR in human leukocytes, but not until recently CFTR protein was detected in neutrophils [4,5], in lymphocytes [6,7] and in monocytes [8,9], in which it was shown to be involved in membrane potential regulation [8]. Monocytes can differentiate into macrophages and possible roles of CFTR in those cells include involvement in inflammation [10] and in bactericidal activities [11,12].

Class I mutations represent approximately 10% of total CFTR mutations associated with CF disease in patients worldwide [13]. In CF patients with this class of mutations the presence of a premature stop codon causes the translation of mRNA to cease prematurely. The resulting truncated CFTR

protein does not reach the cell membrane causing a severe impairment of chloride transport in epithelial cells. When exposed to corrector drugs, designed to overcome the defective codon readthrough by ribosomes, increased CFTR protein synthesis can be expected in cells. To test the efficacy of those drugs, variations of CFTR expression levels in easily accessible cells like peripheral blood leukocytes would be valuable. Semi-quantitative information on CFTR expression levels, as well as data of the molecular mass of detected proteins, can be obtained by western blot (WB). Nevertheless, this technique has limited utility in a monitoring program of CF patients due to complex processing of the sample and the relatively great amounts of biological material necessary to perform the analysis. On the contrary, flow cytometry (FC) analysis of cells performed on a few hundred microliters of peripheral blood may represent a very sensitive technique useful to quantify the relative number of CFTR molecules expressed in representative cells as well as their variations.

The aim of this study is to evaluate the potential use of FC analysis in detecting the presence and in measuring relative CFTR protein levels in leukocytes. To our knowledge, only one study has recently analyzed the expression of CFTR in peripheral blood leukocytes by flow cytometry [9]. However, lymphocytes were not studied and CFTR expression was investigated only in a few individuals. In order to test a setting closer to clinical applications we first evaluated the presence of CFTR in peripheral blood mononuclear cells (PBMCs). We then optimized two FC parameters for the comparison of CFTR expression levels in major leukocyte sub-populations in peripheral blood samples from groups of at least five CF patients representing the most frequent *CFTR* mutation classes. Moreover, we tested the sensitivity of FC parameters by analysing variations of CFTR expression levels induced by exposing monocytes from CF patients with nonsense mutation genotype to PTC124 (Ataluren), a drug able to promote the expression of CFTR in a CF mouse model with a CFTR-G542X nonsense mutation [14] and that is under clinical evaluation in a phase III study. The results of FC analysis were related to those of a CFTR functional assay performed in parallel on replicate samples from the same CF patients.

MATERIALS AND METHODS

Patients

Forty CF patients were grouped according to the class of CFTR mutations [2]. The control group (CTRL) consisted of 12 individuals selected among healthy volunteers. Additional control subjects included 12 healthy CF carriers (CFC), i.e. patients' parents, with one mutated *CFTR* allele. Details about CFC and CF patients are shown in **Table 1**. Samples and data were used for analysis only after informed consent was obtained according to guidelines approved by the local Ethical Committee.

Detection of biotinylated CFTR in peripheral blood mononuclear cells (PBMCs)

Roughly $4x10^7$ PBMCs recovered from 25 mL buffy-coat samples were obtained by centrifugation on Ficoll gradient (GE Healthcare, Piscataway, USA). Cells were washed and then incubated for 30 minutes at +4°C with 2mM EZ-link® sulfo-NHS-biotin (Pierce Biotechnology, Rockford, U.S.A.). Excess biotin was removed by three washes with PBS containing 100mM glycine. Total cell proteins were extracted as described previously [8]. A quantity of 65 µg total proteins was immunoprecipitated using protein G sepharose (GE Healthcare, Piscataway, USA) and either of two different rabbit anti-CFTR antibodies (Alomone Labs, Jerusalem, Israel and Santa Cruz Biotechnology, Dallas, U.S.A.) recognizing the C- and N-terminus of CFTR, respectively. Either of two other different rabbit antibodies were utilized to control both efficiency and specificity of the immunoprecipitation procedure. Immunoprecipitates were denatured for 20 minutes at 40°C in Laemmli sample buffer, separated on a 6% acrylamide/bisacrylamide gel and transferred onto nitrocellulose membrane (GE Healthcare, Piscataway, U.S.A.). Membranes were washed, blocked and then incubated with 40 ng/mL HRP-conjugated streptavidin (Jackson Immunoresearch Laboratories, West Grove, U.S.A.), which binds to biotinylated proteins. Signal was detected on nitrocellulose membrane by the HRP-catalyzed ECL method (Millipore, Billerica, U.S.A.).

Immunofluorescence labelling of leukocytes

Aliquots of 100 µL peripheral blood were used for FC analysis within five hours after blood withdrawal. Red blood cells were lysed in 1 mL solution containing: 0.89% (w/v) NH_4Cl, 0.10% (w/v) $KHCO_3$ and 200µM EDTA. After addition of 5% FBS in PBS, samples were centrifuged for 7 min at 300x*g* at room temperature, the supernatant was reduced to a volume of 100 µL and 10 µL human serum (Sigma-Aldrich, Missouri, U.S.A.) was added. Cells were incubated with a mouse anti-CD14 antibody conjugated with the tandem fluorophores PE-Cy7(λ_{nm} = 488_{ex}/>750_{em}) or with allophycocyanin (APC) fluorochrome (λ_{nm} = 635_{ex}/670_{em}) according to manufacturer (BioLegend, San Diego, U.S.A. and Miltenyi Biotech, Germany, respectively). Washed cells were fixed for 20 minutes at room temperature in 0.5 mL Fixation buffer (BioLegend, San Diego, U.S.A.). After treatment with Permeabilization Wash Buffer (PWB) according to manufacturer (BioLegend, San Diego, U.S.A.), cells were incubated (45 min at room temperature) with primary antibody (polyclonal rabbit anti-CFTR Alomone Labs, Jerusalem, Israel, 800 ng or 150 ng/100 µL blood sample from lot numbers 04 and 05, respectively). To decrease non specific binding, human serum (10% v/v) was added to sample before its incubation with primary antibody. To measure the contribution of non specific antibody-cell interactions the rabbit polyclonal primary antibody was pre-incubated with a blocking peptide (4 µg) corresponding to aminoacids 1468-1480 in the C-terminal domain of CFTR. A mouse monoclonal anti-CFTR antibody, clone 13-1 (1.2 µg/sample) from R&D System, Minneapolis, U.S.A., was used in some replicate samples. This monoclonal antibody recognizes an epitope on the R-domain of CFTR. Goat anti-Rabbit IgG antibody (1.5 µg/sample) conjugated with Alexa Fluor (AF) 488 (Life Technologies, Carlsbad, U.S.A.) or Rabbit $F(ab')_2$ anti-Mouse Ig antibody (300 ng/sample) conjugated with FITC (DAKO, Glostrup, Denmark) were used as secondary antibodies.

Flow cytometric (FC) analysis of CFTR expression in leukocytes and effect of PTC124

Fluorescence, forward and side scatters were determined in cells using a MACSQuant Analyser (Miltenyi Biotech, Cologne, Germany) processing 80000 total events and performing statistics of roughly 2000 monocytes with FCS Express v3 software (De Novo Software, Los Angeles, U.S.A.). Granulocyte, lymphocyte and CD14 positive monocyte cell populations were identified and gated by visual inspection of dot plots using side scatter values versus CD14/PE-Cy7 fluorescence values. A threshold value (background) was determined in sample stained with rabbit polyclonal anti-CFTR antibodies pre-incubated with blocking peptide. The percentage of events above the threshold corresponded to background noise. The percentage of CFTR positive cells (%CFTR positive cells) was calculated as the difference between the percentage above the threshold obtained without blocking peptide and background noise. The ratio between Mean Fluorescence Intensity (MFI) values in samples processed with and without blocking peptide was related to CFTR expression levels in cells. Average MFI ratio values and average %CFTR positive cells values were used to compare different groups of individuals.

Flow cytometric analysis of CFTR expression levels was performed using also enriched monocytes (>70% purity) from 6 CF patients carrying a *CFTR* nonsense mutation. Results obtained from monocytes exposed *ex vivo* for 24 hours to 10μM PTC124 (Selleckchem, Houston, U.S.A.) were compared to untreated controls (CTRL). This small-molecular agent has been designed by PTC Therapeutics to make ribosomes less sensitive to premature stop codons. Fixed cells were stained using the polyclonal rabbit anti-CFTR antibody, as described above. A total of 10.000 events corresponding to purified cells were registered and analysed for CFTR expression levels.

Functional CFTR assay

CFTR function was tested by imaging cell membrane potential of adherent monocytes using the probe bis-(1,3-diethylthiobarbituric acid) trimethine oxonol ($DiSBAC_2(3)$, Life Technologies, Carlsbad, U.S.A.), as previously described [8]. In this assay, CFTR function is expressed by a CF

index, which has positive values when CFTR anion channel functions properly and negative values when its ability to transport chloride anions through plasma membrane is reduced or absent.

Sub-cellular localization and quantification of CFTR by confocal microscopy

To localize the cellular distribution of fluorescent probes corresponding to CFTR and CD14 molecules, leukocytes already labelled with antibodies as described above were stained also with 3μM DAPI (Sigma-Aldrich, Missouri, U.S.A.). Cells were plated onto microscopy slides, dried and cover slip was added with anti-fading mounting medium. They were then analysed with a Leica TCS-SP5 confocal microscope (Leica Microsystem, Wetzlar, Germany). Wavelengths were the same as those selected in flow cytometric studies whereas for DAPI the following wavelengths were used: $\lambda_{nm} = 360_{ex}/460_{em}$. Z-stack images were merged to quantify CFTR signal in cells. Gray scales of 32-bit TIFF images were measured with ImageJ ver 1.43u. Around 10 cells/sample were analysed and background values were subtracted. Mean values of cells from each CF patient and each healthy CTRL donor were used to compare the two groups.

Statistics

SigmaStat software version 3.0 (SPSS Inc., Chicago, U.S.A.) was used for statistical calculations. According to the distribution of results, parametric and/or nonparametric statistics were applied to test hypotheses on the means and/or the medians. The α value was set to 0.05 and the level of significant difference to $P < 0.05$.

RESULTS

Cell membrane localization of CFTR in peripheral blood mononuclear cells

CFTR expression in non CFTR-defective PBMCs is illustrated in **Figure 1.** Two different forms of biotinylated CFTR (lanes 2 and 4) were immunoprecipitated (IP) by either of two rabbit anti-

CFTR antibodies recognizing the C-terminus or N-terminus of CFTR. The two antibodies may have different IP efficiency, as suggested by the different signal intensities of common bands detected at around 150 and 180 kDa. The specificity of the result is suggested by the absence of bands in the same sample immunoprecipitated with either of two non CFTR-related rabbit polyclonal antibodies (**Figure 1**, lanes 1 and 3). **Figure 2** shows the different expression of CFTR in leukocytes from healthy CTRLs (A-D) and from two CF patients with genotypes R1158X/E585X (E-G), and W1282X/F508del (H). Leukocytes were stained with rabbit polyclonal anti-CFTR antibody (**Figures 2D and H**) or with mouse monoclonal anti-CFTR antibody (**Figures 2B and F**) and anti-CD14 antibody (**Figures 2A and E**). A partial co-localization of CFTR and CD14 on monocyte plasma membrane is shown in **Figure 2C**. A more intense green fluorescence, corresponding to CFTR staining, was observed in monocytes from healthy CTRLs (**Figures 2B and D**) than in monocytes from CF patients (**Figures 2F and H**) whereas the red fluorescence intensity of CD14 staining is similar in monocytes of CTRLs and CF patients. This was observed in results obtained by the polyclonal as well as by the monoclonal antibodies. Histogram in Figure 2 summarize the quantification data of CFTR signal in cells from three healthy CTRL donors and from three CF patients. A paired t-test revealed a significant difference between the mean values of the two groups (P=0.018).

Setting parameters for FC analysis of whole blood samples: MFI ratio and percent of CFTR positive cells

Figure 3 illustrates how sub-cellular populations of CFTR positive leukocytes were identified by FC analysis in two whole peripheral blood samples stained with anti-CD14 and with polyclonal rabbit anti-CFTR antibodies. A sub-population of CD14 positive cells, different from lymphocytes and granulocytes that stained very weakly with anti-CD14 antibody, can be easily distinguished plotting side scatter versus PE-Cy7 fluorescence intensity values (**Figure 3A**). This highly CD14 positive sub-population with relatively low side scatter values was assigned to monocytes. **Figures**

3B and D illustrate the positioning of thresholds for CFTR positivity in monocytes from a healthy CTRL (**Figure 3B**) and from a CF patient (**Figure 3D**) homozygous for a class I *CFTR* mutation (R1162X). In the presence of blocking peptide (**Figures** 3B and D) less than 10% CTRL and CF monocytes incubated with rabbit polyclonal anti-CFTR antibody had AF488 fluorescence detected above the threshold. Without blocking peptide (**Figures 3C and E**) those percentages increased to about 80% in monocytes from healthy CTRL (**Figure 3C**) and only to about 40% in monocytes from CF patient (**Figure 3E**). Thresholds positioned in the same way were utilized to distinguish CFTR expression from the background noise in lymphocytes. The ratio between MFI value of the replicate sample processed without blocking peptide and the MFI value of the corresponding replicate sample processed with blocking peptide reflected CFTR expression in sub-populations of leukocytes being analysed.

Reproducibility and robustness of FC analysis

The same operator labelled and analyzed different blood withdrawals from the same subject to test the repeatability of the FC analysis. **Table 2** summarizes the different test conditions and results. Intraindividual biological and technical (eg. different batches of reagents) variabilities were tested by separately analysing blood withdrawals, obtained within a period of two years, from two subjects. Samples from one of these subjects (number 2, **Table 2**) were also analysed frequently during this period, and results obtained with either of two different anti- CFTR antibody batches (lots 04 and 05) are presented in order to highlight the contribution of this parameter to the overall variability. The within-day repeatability was tested twice (Day A and Day B, **Table 2**) by performing the labelling and analysis in parallel of four replicates using the same blood sample and the same reagents. The analytical variability decreased significantly when testing the within-day repeatability, as shown by the coefficient of variation (CV) of MFI ratio values (**Table 2**) resulting 1.6% and 2.8% in the two tests performed, respectively.

To test the reproducibility of the FC test, blood samples from the same subject were labelled and analyzed repeatedly (n=6) during a period of ten months by two operators not working in parallel. Operator 1, who performed the tests of repeatability above, obtained the following results; (MFI ratio: mean = 1.88, CV = 11.0% and %CFTR positive monocytes: mean = 84.2, CV = 5.8%) Operator 2, who performed this FC analysis less frequently, obtained the following results; (MFI ratio: mean = 2.11, CV = 17.3% and %CFTR positive monocytes: mean = 82.0, CV = 12.6%).

To test how robust the FC analysis was, a comparison was performed on mean values (mean±SD) of MFI ratio and of %CFTR positive monocytes in blood samples from healthy CTRLs, from CFC healthy individuals and from CF patients. Patients were grouped according to their membership in four different *CFTR* mutation classes: I+I (n = 12), I+II (n = 12), II+II (n = 11), and I+V (n = 5). ANOVA analysis was followed with Holm-Sidak method and the results, summarized in **Figure 4,** show that monocytes of all CF patient groups had significantly lower average MFI ratio values than monocytes of healthy CTRLs. Average MFI ratio value was significantly lower in monocytes of CF patients with class I mutations on both alleles (**Figure 4 A**, I+I) than in monocytes of healthy CFC individuals, but no significant difference was observed among groups of CF patients or between healthy CTRLs and healthy CFC individuals. It may be noted that interindividual variability, as indicated by CV%, was comparable among groups and also comparable to the intraindividual variability shown in **Table 2**. On the contrary, greater variability was observed among values indicating %CFTR positive monocytes in some groups, as shown in **Figure 4 B**. Nevertheless, the results of Holm-Sidak method indicated that mean values of %CFTR positive monocytes were significantly different in the group of healthy CTRLs, or in the group of CFC individuals, and groups of CF patients, but differences were also observed among some CF groups as indicated in **Figure 4 B**.

MFI ratio values reflecting CFTR expression in lymphocytes were not normally distributed and significant differences identified among groups by Kruskall-Wallis ANOVA on ranks (P = 0.003) were not further indicated by multiple pair-wise comparisons performed with Dunn's method. The

%CFTR positive lymphocyte mean values resulted significantly different among groups (ANOVA P<0.03). In this case, pairs-wise comparison by Holm-Sidak method identified differences between; healthy CTRLs (38.9±13.3) and class I+II CF patients (16.0±12.8, P = 0.004), CFC individuals (26.7±19.2) and class II+II CF patients (45.4±21.9). Class I+II CF patients with the lowest average %CFTR positive lymphocyte values resulted significantly different also from classes II+II (P<0.001), I+I (32.2±19.1, P = 0.004) and I+V (42.8±17.8, P = 0.004) CF patients.

However, the high CV% values ranging from 34% in CTRLs to 80% in the class I+II group reflect an important contribution of interindividual variability when %CFTR positive lymphocytes were compared.

Detection of drug-induced CFTR expression variations in monocytes by FC analysis

Type I mutations are expected to severely impair CFTR expression in CF patients. The CFTR corrector PTC124 (Ataluren) is currently under clinical evaluation for its potential to restore CFTR protein expression and function as chloride channel. In order to properly associate the results of FC analysis to a genuine drug response, FC analysis and a CFTR functional assay were performed in replicate samples of monocytes from 6 CF patients with different CFTR genotypes (all including a CFTR nonsense mutation) by two researchers unaware of the other's results. The results are summarized in **Table 3**. In monocytes from patients #1 and #2, exposed *ex vivo* for 24 hours to 10μM PTC124, the MFI ratio values increased 25% and 12%, respectively, as compared to control samples (0.1% DMSO in cell culture medium). These variations are greater than those measured in within-day replicates (CV% <3%, see above and **Table 2**). Functional assay run in parallel showed a recovery of CFTR function in the same samples. In monocytes from the remaining four patients CFTR expression levels were unchanged (differences between treated and untreated samples were lower than 3%). Interestingly, we did not detect any improvement of CFTR function either in these same four samples exposed to PTC124.

DISCUSSION

There is increasing evidence that myeloid cells may play an important role in the pathogenesis of CF [15]. Therefore, the measurement of CFTR expression in easily accessible peripheral blood leukocytes has important implications, not only as surrogate biomarker but also as a way to monitor the correction of the defect in cells that are key players in CF. This monitoring may be of particular importance because drugs targeting defective CFTR are already available and others are in advanced stages of clinical evaluations for the therapy of CF patients [16]. Different methods to evaluate the effects of CFTR corrector drugs in patients suffer from some objective limitations and the availability of a reproducible analytical method capable of detecting CFTR expression in human leukocytes might represents an important advance in this context.

The localization of CFTR protein on the plasma membrane of peripheral blood leukocytes was verified by a cell surface protein biotinylation approach of intact cells. Combining immunoprecipitation, gel electrophoresis and streptavidin detection of biotinylated proteins we revealed a band at around 150 kDa that was thicker than that at around 180 kDa, which is the predicted molecular mass of fully glycosylated and mature form of the CFTR protein (**Figure 1, lanes 2 and 4**). This 150 kDa band may represent both the product of a partial degradation of the CFTR protein during or after the biotinylation process as well as a differentially processed form of CFTR, as indicated previously [8]. A role of calpain in the turnover of membrane bound CFTR has already been suggested in PBMCs [17]. However, relative masses described for calpain-derived CFTR fragments were 70 kDa and 100 kDa, respectively. Therefore the 150 kDa band should not represent the product of CFTR digestion by calpain. CFTR protein partially co-localizes with CD14 antigen on the plasma membrane of monocytes as revealed by confocal microscopy studies. Therefore we set conditions to detect CFTR expression in peripheral blood leukocytes by flow cytometry. While we could detect fluorescence related to CFTR expressed in monocytes and lymphocytes, the possibility to detect a specific CFTR signal in granulocytes was precluded in our study, probably because the permeabilization of plasma membrane induced high levels of auto-

fluorescence in those cells (data not shown). Granulocytes, which represent the most abundant cell population in peripheral blood, have been reported to express CFTR [4,5]. However, altered CFTR protein expression levels in neutrophils have been related to inflammation [4]. Also absent CFTR expression as detected by FC in neutrophils has been reported [9], but low signal to noise ratio values might not have been properly considered and the contribution of high MFI values obtained with an isotype control antibody may have been underestimated. When we analysed CFTR expression in lymphocytes, the second largest sub-population of leukocytes in peripheral blood samples, we noticed low autofluorescence after cell permeabilization making lymphocytes more suitable than granulocytes for CFTR expression analysis by FC. Moreover, some significant differences could be observed comparing MFI ratio values and the percentages of CFTR positive lymphocytes among groups. Unfortunately, those results were strongly influenced by a high degree of variability, possibly related to different CFTR expression in lymphocyte sub-populations (e.g. B- and T-cells). Specific biomarkers can be used to separate cell sub-populations, but they both impact the costs of the test and increase the complexity of interpretation of the results. Therefore, we focused on the optimization of MFI ratio values and %CFTR positivity in monocytes that, in our hands, represented the most suitable leukocyte sub-population for FC analysis of CFTR expression in peripheral blood.

Both analytical and biological variability contributes to the results of ANOVA performed to distinguish differences among groups of CF patients, healthy CFC and healthy CTRL individuals according to the levels of CFTR expression in monocytes. In order to limit analytical variability we pre-incubated the primary antibody with a blocking peptide instead of using an isotype control antibody to calculate MFI ratio values. In this way we reduced the experimental variability, for instance caused by use of different antibody lots or by stability over time of fluorescent probes ensuring that significant differences identified by ANOVA preferentially reflected biological rather than analytical variability. The MFI ratio values of CFTR staining in monocytes showed lower variability as compared to those indicating %CFTR positive monocytes, as suggested by the values

of CV% (**Figure 4**). This may be partially explained by the adjustment of the threshold value that is necessarily set experimentally during the analytic phase of the assay. Small changes of this threshold (between 5 to 10% of gated events) negatively impact on the results of the statistical analysis when groups of samples from different individuals are considered.

We further wanted to test the capability of FC analysis to detect changes of CFTR expression levels in monocytes exposed *ex vivo* to PTC124 (Ataluren), a CFTR corrector under evaluation in clinical trials. As compared to untreated controls, monocytes exposed to PTC124 showed variations greater than those of within-day repeatability in samples from patients #1 and #2, thus suggesting that FC analysis in monocytes can be a useful method to monitor variations of CFTR expression levels related to drug treatment. Monocytes from the same blood sample showed a significantly increased CFTR function after exposure to PTC124, thus proving that the response to the drug measured by FC was reflecting a true recovery of CFTR expression on the cell membrane. This result interpretation was further supported by the evidence that the variations of CFTR expression in monocytes from patients # 3-6 were similar to the within-day repeatability of controls (1-2%) and that the results of the functional CFTR assay performed on the same samples documented the inability of PCT124 to induce any significant improvement of CFTR activity in those four samples. It is still not clear whether the responses to protein translation correctors might be influenced by an individual capability of ensuring a proper drug mediated read-through of the mutation by the translation machinery. A possible explanation of the absence of PTC124 effects in four cases out of six may be an absence of substrate due to an increased degradation of mRNA containing a premature stop codon, as discussed by Linde and Kerem [18]. Being the patients heterozygous for a nonsense mutation, another possible influence might derive from the presence of the second mutation that might impact on the capability to respond to the treatment. Variability of responses to drugs in cells and patients are common occurrence and the observed concordance between expression and the functional assays allows us to conclude that FC analysis might represent a powerful approach for the evaluation of responses to drugs targeting specific basic defects in CF

leukocytes. However, we realize that the robustness of this analytical approach must be confirmed in a greater number of observations either *ex vivo* or *in vivo* before proposing its use in the follow-up of CF patients.

In conclusion, we have described a relatively simple method to test CFTR expression in leukocytes starting from a few milliliters of peripheral blood that can be easily obtained from patients without too much discomfort. Once validated, the test might represent a convenient approach for the monitoring of selected therapies in CF patients.

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Figure legends

Fig. 1. Detection of CFTR in peripheral blood mononuclear cells (PBMCs). After biotinylation of intact PBMCs, protein extracts were immunoprecipitated with either of two different rabbit anti-CFTR antibodies recognizing the C-terminus (lane 2) or the N-terminus (lane 4) of CFTR and compared with samples incubated with either of two different rabbit control antibodies (lanes 1 and 3, respectively). Proteins separated on 6% acrylamide gel were transferred onto nitrocellulose membrane and biotinylated proteins were detected with HRP-conjugated streptavidin and ECL. In lanes 2 and 4, two bands detected at around 150 kDa and at 180 kDa were assigned to CFTR.

Fig. 2. Confocal analysis of CFTR expression in leukocytes.

Upper panel. Leukocytes from a healthy individual (A-C) and from a CF patient with genotype R1158X/E585X (E-G). A and E: cells stained with APC-conjugated anti- CD14 antibody, fixed and then stained in B and C with a mouse monoclonal (clone 13-1) anti-CFTR and FITC-conjugated secondary antibodies (B and F). Merged images are shown in C and G. The green fluorescence of a CFTR positive monocyte from a healthy CTRL individual is shown in B; a granulocyte and a CD14 positive monocyte are shown in E. Leukocytes from a healthy CTRL in D and from a CF patient (genotype W1282X/F508del) in H were stained with a polyclonal rabbit anti-CFTR antibody and detected with an AF488-conjugated secondary antibody. Nuclei were stained with DAPI (blue) and shown in all images, except in B and in F. Scale bar = 10 μm.

Lower panel: Quantification of fluorescence signals in cells from healthy CTRL donors and from CF patients (two patients with I+I and one patient with I+II mutation classes). Bars are means±S.E.M., paired t-test, P=0.018, n=3.

Fig. 3. Flow cytometry analysis of dot-plot graphs of human leukocytes in whole blood samples of healthy CTRLs. A. The separation of granulocytes, monocytes and lymphocytes sub-populations by

plotting side scatter vs. fluorescence emitted by PE-Cy7 fluorochromes (CD14, monocytes). Forward scatter values of gated monocytes from a healthy CTRL (B and C) and a CF patient with genotype R1162X/R1162X (D and E) plotted vs CFTR protein signal detected in green channel by AF488 fluorochrome. The threshold of CFTR-signal (red line) was set to obtain 90-98% total monocytes, stained with anti-CFTR antibody pre-incubated with blocking peptide , below the value (B and D). Cells stained with anti-CFTR antibody without blocking peptide are shown in C and E, respectively. To calculate the percentage of CFTR positive monocytes the values in B (or in D) were subtracted from values in C (or in E), respectively (eg. 83.4 – 5.0 = 78.4%).

Fig. 4. FC analysis of CFTR expression data in monocytes. Box and whisker plots (25^{th}-75^{th} percentiles) of MFI ratio values (A) and percent CFTR positive monocyte values (B) in healthy controls (CTRLs), healthy CF carriers (CFC) and cystic fibrosis (CF) patients grouped according to *CFTR* mutation class (I, II and V) being carried. The midline in boxes indicates median and whiskers represent the lowest and highest values, respectively. Symbols indicate significant differences (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$) identified by Holm-Sidak method used to compare means of pairwise groups.

Table 1. Subjects with known genotype enrolled in the study.

#	Mutation class	Gender	Age (years)	Genotype	
Healthy carriers of a CFTR mutation (CFC)					
1	I	F	48	WT	G542X
2	I	F	39	WT	R1162X
3	I	M	47	WT	R1162X
4	I	F	44	WT	R1162X
5	I	F	62	WT	4013insT
6	I	F	61	WT	R1162X
7	I	F	58	WT	1717-1G>A
8	I	M	52	WT	2183AA>G
9	I	F	56	WT	R1162X
10	I	M	71	WT	W1282X
11	II	F	38	WT	F508del
12	II	F	46	WT	F508del
CF patients					
13	I + I	M	17	G542X	G542X
14	I + I	M	26	R1162X	R1162X
15	I + I	M	33	R1162X	R1162X
16	I + I	M	35	W1282X	621+1G>T
17	I + I	F	13	R1162X	R1162X
18	I + I	M	20	E585X	R1158X
19	I + I	F	44	R1162X	R1162X
20	I + I	F	25	G542X	R553X
21	I + I	F	36	Q552X	CFTRdele17a-18
22	I + I	M	37	G542X	CFTRdele1
23	I + I	M	31	Q552X	2183AA>G
24	I + I	M	12	R1162X	R1162X
25	I + II	M	18	Q39X	F508del
26	I + II	F	20	G542X	F508del
27	I + II	F	42	R1162X	F508del
28	I + II	F	23	R1162X	F508del
29	I + II	F	28	W57X	F508del
30	I + II	M	28	Q552X	F508del
31	I + II	M	14	R1162X	F508del
32	I + II	F	25	W1282X	F508del
33	I + II	M	11	G542X	F508del
34	I + II	M	16	Q552X	F508del
35	I + II	F	25	R1162X	F508del
36	I + II	F	23	R1162X	F508del
37	I + V	M	43	R1162X	3849+10kbC>T
38	I + V	F	32	R1162X	2789+5G>A
39	I + V	F	38	R1162X	3849+10kbC>T
40	I + V	M	46	G542X	3849+10kbC>T
41	I + V	F	24	R1162X	3849+10kbC>T

42	II + II	M	40	F508del	F508del
43	II + II	F	37	F508del	F508del
44	II + II	F	21	F508del	F508del
45	II + II	F	38	F508del	F508del
46	II + II	M	17	F508del	F508del
47	II + II	M	42	F508del	F508del
48	II + II	F	22	F508del	F508del
49	II + II	M	43	F508del	F508del
50	II + II	F	42	F508del	F508del
51	II + II	F	29	F508del	F508del
52	II + II	M	13	F508del	F508del

Table 2.

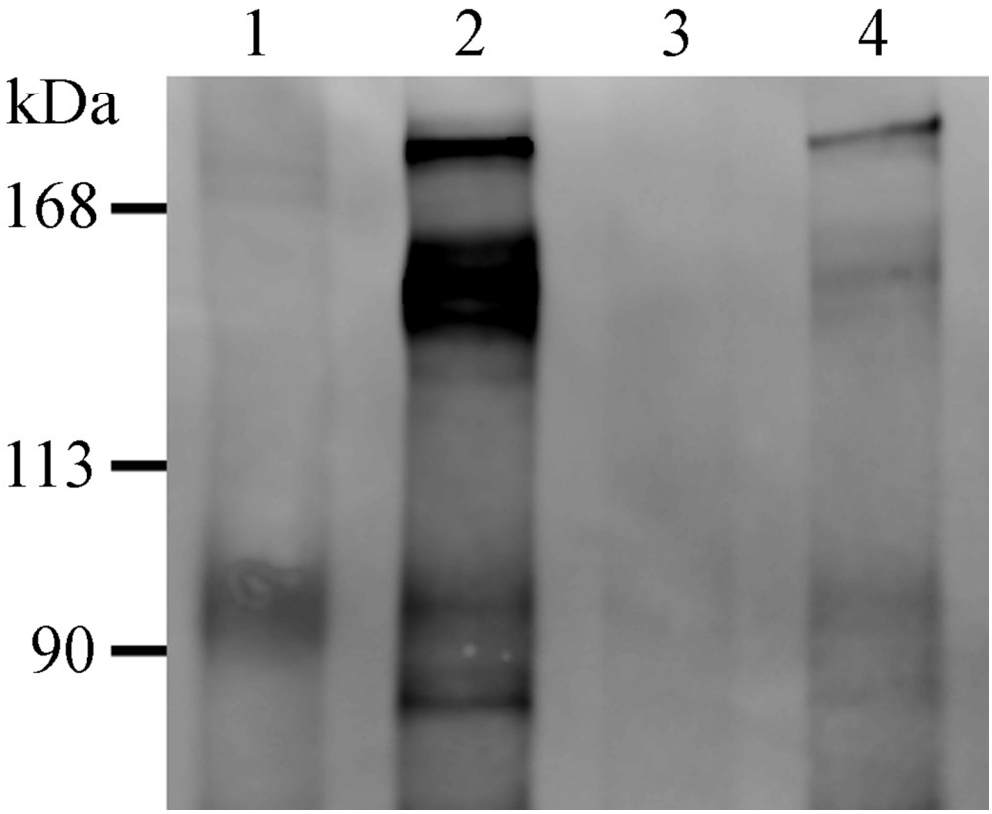
	n	MFI ratio		%CFTR positive monocytes	
		Mean	CV	Mean	CV
Subject 1[i] (♀)	6	1.88	11.0%	84.2	5.8%
Subject 2[i] (♂)	6	1.95	13.1%	83.7	13.9%
Lot 04[ii] (♂)	21	2.13	21.7%	82.7	12.8%
Lot 05[ii] (♂)	36	1.82	10.3%	74.8	14.6%
Day A[iii] (♂)	4	1.68	1.6%	62.8	10.5%
Day B[iii] (♂)	4	1.64	2.8%	55.2	9.9%

Table 2. Repeatability of FC analysis of CFTR expression in monocytes. i) The same operator repeated FC analysis six times of samples from the same control individuals in different days; ii) between-day repeatability was tested by analyzing different samples from the same individual with either antibody Lot 04 or Lot 05; iii) within-day repeatability was tested by analyzing one sample four times on the same day and using the same antibody Lot.

Table 3.

ID #	Gender	Genotype	MFI before PTC124	MFI after PTC124	Variation (%CTRL)	CF index CTRL	CF index after PTC124	Variation (%)
1	F	R1162X/ 3849+10kbC>T	1.47	1.84	**+25**	-41	+16	+57
2	M	R553X/ D1152H	2.17	2.44	**+12**	-74	-33	+41
3	F	R1162X/ N1303K	2.78	2.79	**+1**	-36	-32	4
4	M	S466X/ R1070Q/ 1717-1G>A	1.76	1.72	**-2**	-2	-22	-20
5	M	G542X/ 3849+10kbC>T	1.67	1.65	**-1**	-70	-95	-25
6	M	Q552X/ 2183AA>G	2.24	2.28	**+2**	-25	-64	-39

Table 3. MFI ratio values and CF index variations induced in monocytes exposed *ex vivo* for 24 hours to 10 μM PTC124 CFTR corrector or to 0.1% (v/v) DMSO (CTRL). Mutations being targets for PTC124 are underlined. CFTR expression levels and CFTR function reflected by MFI ratio and CF index values, respectively, were obtained by two researchers processing replicate samples of monocytes in parallel and unaware of the other's result. An increased CFTR function results in an increment of CF index value.



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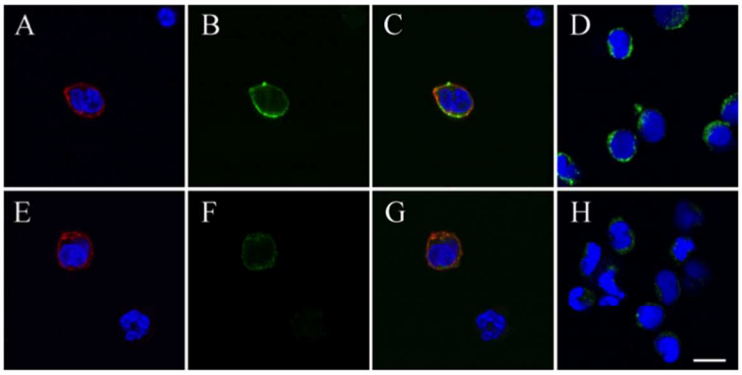

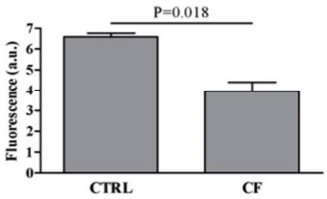

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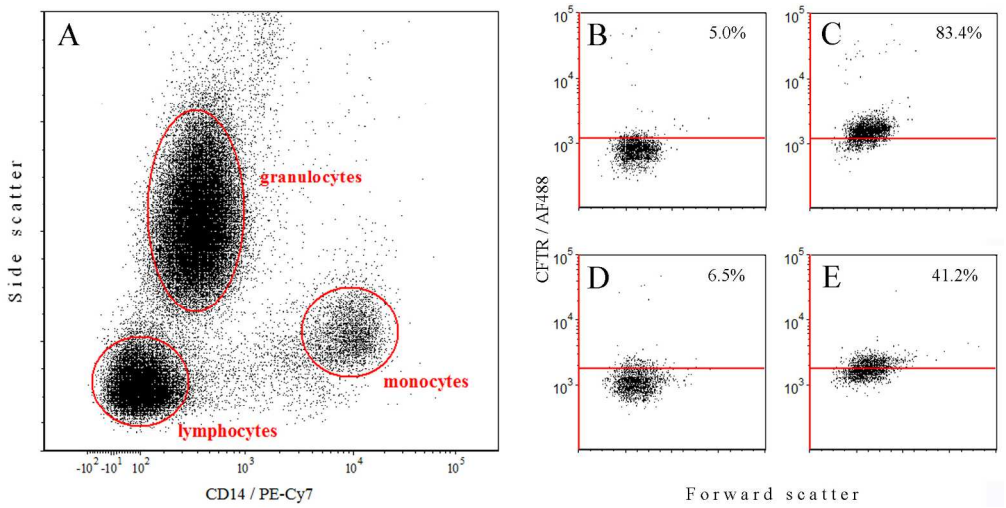

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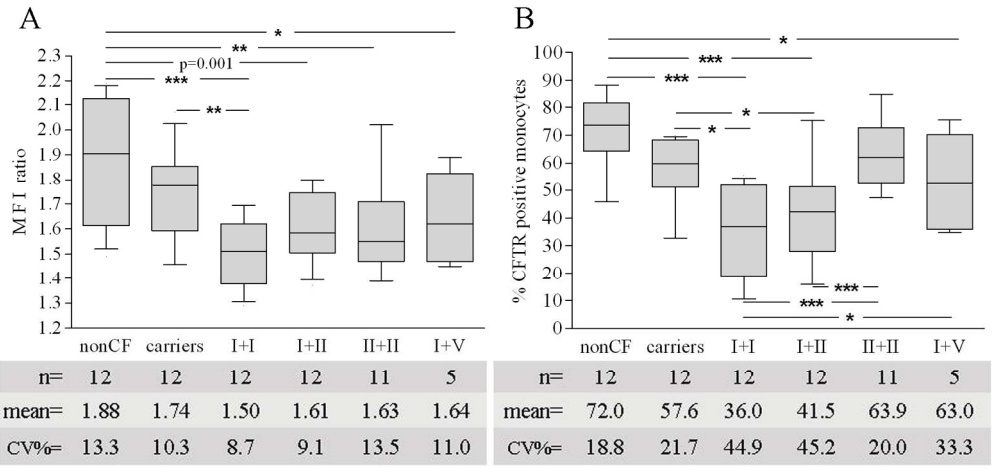

	nonCF	carriers	I+I	I+II	II+II	I+V
n=	12	12	12	12	11	5
mean=	1.88	1.74	1.50	1.61	1.63	1.64
CV%=	13.3	10.3	8.7	9.1	13.5	11.0

	nonCF	carriers	I+I	I+II	II+II	I+V
n=	12	12	12	12	11	5
mean=	72.0	57.6	36.0	41.5	63.9	63.0
CV%=	18.8	21.7	44.9	45.2	20.0	33.3

161x76mm (300 x 300 DPI)

Table 1. Subjects with known genotype enrolled in the study.

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Table 2. Reproducibility of FACS analysis of CFTR expression in monocytes. i) The same operator repeated 6 times FACS analysis on the same control individuals in different days; ii) within-days repeatability performed analyzing different samples from the same individual with either antibody Lots 04 or 05; iii) within-day repeatability performed analyzing one sample four times on the same day and using the same Lot of antibody.

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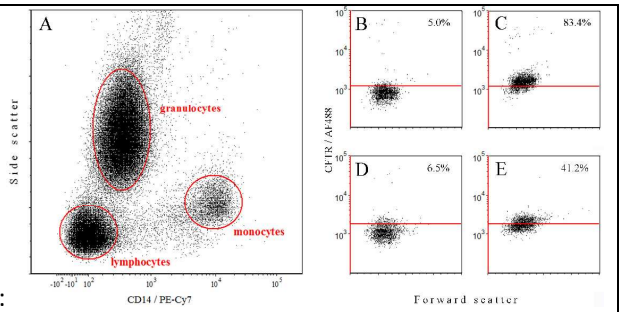
Cytometry Part A
Author Checklist: MIFlowCyt-Compliant Items

Requirement	Please Include Requested Information
1.1. Purpose	Test CFTR expression in human leukocytes and specificity of anti-CFTR antibody
1.2. Keywords	CFTR human leukocytes flow cytometry
1.3. Experiment variables	Biological samples from different human individuals
1.4. Organization name and address	Department of Pathology and Diagnostics, University of Verona, Strada Le Grazie 8, 37134 Verona, Italy
1.5. Primary contact name and email address	Claudio Sorio, claudio.sorio@univr.it
1.6. Date or time period of experiment	2011-2013
1.7. Conclusions	A specific signal of CFTR expression was observed in monocytes and lymphocytes, but not in granulocytes
1.8. Quality control measures	Instrument calibrations with beads were performed
2.1.1.1. (2.1.2.1., 2.1.3.1.) Sample description	Human samples with known *CFTR* genotypes
2.1.1.2. Biological sample source description	Peripheral whole blood
2.1.1.3. Biological sample source organism description	Homo sapiens
2.1.2.2. Environmental sample location	EDTA treated polystyrene test tubes
2.3. Sample treatment description	Erythrocyte lysis, CD14 labelling, 4%PFA fixation, permeabilization, CFTR labelling
2.4. Fluorescence reagent(s) description	Anti-CD14 antibody with PE-Cy7 or APC, secondary antibodies with AF488 or FITC
3.1. Instrument manufacturer	Miltenyi Biotech
3.2. Instrument model	MACSQuant Analyzer
3.3. Instrument configuration and settings	Normal and same detector voltages among samples, optical filters installed Nov 2010
4.1. List-mode data files	*We recommend all authors to submit their data files to http://flowrepository.org and to make them available for the peer-review process. If you have done so, please let us know by inserting the following codes (replace the red text): 1) The link for peer-review process: http://flowrepository.org/id/FR-FCM-ZZ85 2) The repository identifier: http://flowrepository.org/id/FR-FCM-ZZ85
4.2. Compensation description	Tested and found not to be necessary
4.3. Data transformation details	FCS files analysed with FCS Express v3 software (De Novo Software, Los Angeles, U.S.A.).
4.4.1. Gate description	Based on SSC values and CD14-signal
4.4.2. Gate statistics	Geometrical means of MFI values were used

4.4.3. Gate boundaries	 Subjective: 5-10% of gated events below threshold obtained with anti-CFTR antibody preincubated with blocking peptide.

Notes

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